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Genetic Divergence between North American Ancestral Soybean Lines and Introductions with Resistance to Soybean Cyst Nematode Revealed by Chloroplast Haplotype

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Domesticated soybean [*Glycine max* (L.) Merr.] is a major crop with an established ancestral relationship to wild soybean (*Glycine soja* Sieb. & Zucc.) native to Asia. Soybean genetic diversity can be assessed at different levels by identification of polymorphic alleles at genetic loci, in either the plastid or nuclear genomes. The objective of this study was to evaluate genetic diversity based on chloroplast haplotypes for soybean genotypes present in the USDA germplasm resource collection. Shared chloroplast haplotypes represent broad groups of genetic relatedness. Previous work categorized three-quarters of the cultivated soybeans from Asia into a single haplotype group. Our results confirmed the close relationship of North American soybean ancestors and *G. max* plant introductions previously identified as representing potential sources of soybean genetic variation with the finding that these genotypes belonged to a single chloroplast haplotype group. Genetic diversity was identified in soybean genotypes determined to have a high density of single nucleotide polymorphisms and in a screen of accessions with resistance to soybean cyst nematode. Characterization of soybean plant introduction lines into chloroplast haplotype group may be an important initial step in evaluating the appropriate use of particular soybean genotypes.

Introduction

Soybean [*Glycine max* (L.) Merr.] is a major crop with a significant contribution to food production from the quality protein and abundant oil present in mature seed. Plant breeders continue to release improved cultivars with enhanced yield, disease resistance, and quality traits. One objective of a breeding program is to incorporate complex traits into existing breeding material by using a range of germplasm sources. It has been well documented that the genetic base of North American soybean cultivars is extremely narrow, with 12 progenitor genotypes being responsible for almost 80% of the ancestry of current cultivars (Gizlice et al. 1994; Sneller 1994). The narrow genetic base of current soybean cultivars may lack sufficient allelic diversity to counteract vulnerability to shifts in environmental variables. An investigation of genetic relatedness at a broad level may provide important information about the historical relationship among different genotypes. The USDA Germplasm Resources Information Network (GRIN) (www.ars-grin.gov/npgs) maintains a large collection of soybean germplasm and information about the origin and characterization of a variety of traits/phenotypes. However, access to results of a molecular genetic analysis of the germplasm has lagged behind phenotypic and biochemical characterizations.

Plant chloroplast DNA diversity provides an accessible measure with which to compare evolutionary divergence. Chloroplasts are present in high copy number in cells, are maternally inherited, and have a lower tolerance for accumulated mutations than does nuclear DNA (Hatfield et al. 1985; Wolfe et al. 1987; Provan et al. 1999). Genetic relatedness as determined by differences in soybean chloroplast DNA using restriction fragment length polymorphisms (RFLP) with a small set of restriction enzymes and probes was initially reported for soybean cultivars and plant introductions (Shoemaker et al. 1986). Three general categories of soybean chloroplast DNA for *G. max* and *G. soja* were eventually recognized by RFLP and sequencing studies: type I, type II, and type III (Close et al. 1989; Kanazawa et al. 1998; Abe et al. 1999; Shimamoto et al. 2000; Xu et al. 2000, 2001, 2002). The predominant chloroplast type for cultivated soybeans was type I, whereas smaller

subsets of cultivated soybeans belonged to type II and type III groups. Type I and type II chloroplasts are very closely related, with only one nucleotide difference reported (Close et al. 1989; Xu et al. 2000; Sakai et al. 2003). The type III chloroplast group was shown to be more distantly related to the type I and II groups (Xu et al. 2001) and consisted of the vast majority of *G. soja* accessions tested. Recently, molecular evidence for multiple origins of cultivated soybeans in Asia has been presented based on haplotypes determined by SSR (simple sequence repeat) analysis of six chloroplast (cp) loci (Xu et al. 2002). Xu et al. (2002) reported 8 cpSSR haplotypes accounted for all of the 183 *G. max* lines investigated while examples of 52 distinct haplotypes were identified from the 143 *G. soja* accessions tested. Of the eight *G. max* cpSSR haplotypes, a single haplotype (#49) accounted for 75% of the accessions tested. Haplotype #49 corresponded to the type I and type II chloroplast haplotype category, and the remaining 25% of the *G. max* lines with haplotypes other than #49 corresponded to the type III group.

To identify soybeans containing different chloroplast haplotypes as a basis to identify sources of molecular variation in candidate genes, we first relied on other genetic diversity studies rather than a random sample of soybean genotypes. Considerable effort has been put forth to identify sources of soybean genetic diversity and classify genetic relatedness based on nuclear polymorphisms. When cluster analysis was used to determine relatedness of nuclear genomes in North American soybean ancestors and plant introductions, the results revealed 10 clusters and 3 individual outliers (Brown-Guedira et al. 2000). Several clusters consisted of plant introductions that were distinct from the majority of the ancestral lines (clusters D, J, and K). Zhu et al. (2003) surveyed 25 genotypes for single nucleotide polymorphism (SNP) discovery and reported a very limited number of accessions/cultivars of the genotypes analyzed were found to represent most of the identified SNPs. The lines Peking, PI 209332, and Tokyo contained 83% of the identified SNPs, suggesting that these lines are the most diverse.

In addition to the germplasm representing potentially divergent genotypes, it is informative to categorize the cultivars used in the development of public molecular genetic resources, to have a better perspective of how much genetic diversity is represented in these genotypes. The cultivars Williams 82 (Bernard and Cremeens 1988) and Forrest (Hartwig and Epp 1973) represent important molecular genetic resources because the vast majority of expressed sequence tags (ESTs) were made from Williams 82, and a bacterial artificial chromosome (BAC)-based Forrest physical map exists (Marek and Shoemaker 1997; Meksem et al. 2000; Wu et al. 2004; Shoemaker et al. 2002; Vodkin et al. 2004).

Accessions in the soybean germplasm collection have been characterized for a number of important phenotypic traits. Relevant to this work is the set of soybean accessions characterized for various degrees of resistance to different populations of the soybean cyst nematode (SCN, *Heterodera glycines*). SCN is a major yield-limiting pathogen and impacts soybean production areas worldwide. Because the pathogen exists in the field as a genetically diverse population, characterization of soybean accessions for resistance takes into account different SCN populations (previously termed "races" and now identified as HG types; Niblack et al. 2002). Phenotyping soybean accessions for resistance to SCN is complex with resistance reactions classified into different categories based on relative pathogen reproduction. Over 100 soybean accessions have been identified as having at least some resistance to one or more HG types, and an investigation of the genetic relatedness of those accessions was done with nuclear polymorphic markers (Diers et al. 1997; Zhang et al. 1999). In those studies, principle component analysis revealed that most accessions with SCN resistance clustered into a few major groups.

The objective of our study was to evaluate genetic diversity based on chloroplast haplotypes for representatives of North American soybean ancestors, plant introductions from the clusters identified by Brown-Guedira et al. (2000), accessions resistant to SCN, genotypes used for development of public resources, and a sample of *G. soja* plant introductions.

Materials and Methods

All soybean lines described in this work were obtained from the USDA Soybean Germplasm Collection (Urbana, IL), courtesy of Dr. David Sleper (University of Missouri) or kindly provided by Dr. Thomas Kilen (USDA-ARS, Stoneville, MS). DNA was prepared from leaf tissue from one plant of each line using Whatman FTA cards (Clifton, NJ), a Qiagen DNeasy Plant Mini kit (Valencia, CA), or a Promega Wizard Magnetic 96 DNA Plant System (Madison, WI) following the manufacturer's instructions. Primer sequences for six cpSSRs were the same as those described in Xu et al. (2002) and Powell et al. (1995) with fluorophores placed on the 5′ end of forward primers. Primers for gmcp1, gmcp2, and SOYCP were 6-carboxyfluorescein (FAM)-labeled, and primers for gmcp3, gmcp4, and RD19 were tetrachlorofluorescein (TET)-labeled. Two different analyses were carried out: an assay of all six primer sets to assign a chloroplast haplotype based on the numbering of Xu et al. (2002) and an assay with the gmcp1 primer set alone, which was capable of distinguishing genotypes with haplotype #49 from the other *G. max* haplotypes.

Polymerase chain reaction (PCR) amplifications were done either with each primer set individually or in combinations of two primer sets. A typical 20-μl PCR included 10–100 ng template DNA or FTA punch, 40 mM tricine-KOH (pH 8.0), 16 mM KCl, 3.5 mM $MgCl_2$, 3.75 μg/ml bovine serum albumin (BSA), 200 μM dNTPs, 10% dimethyl sulfoxid (DMSO), 0.5 μM each primer, and 0.2× Titanium Taq polymerase (BD Biosciences, Palo Alto, CA). Amplification conditions were 95°C for 5 min, 40 cycles of 95°C for 20 sec, 50°C or 51°C for 20 sec, 72°C for 20 sec, followed by a 10-min step at 72°C. PCR products were diluted and mixed in ratios dependent on the relative amount of product produced (for example 2 μl gmcp4 and gmcp1 combination,

4 µl gmcp3 and SOYCP combination, and 8 µl gmcp2 and RD19 combination, with 6 µl water). In some cases, aliquots of product from single-primer set PCRs were diluted 10-fold in 20 µl water.

An aliquot of 1.5 µl of the diluted product was combined with 3.5 µl Tamara XL 500 standard (3:10 ratio of standard: formamide) and analyzed for product size on a 4.5% Long Ranger gel (Cambrex Bio Science Rockland, Rockland, ME) using Genscan v. 3.1.2 software on an ABI 377 DNA sequencer followed by Genotyper v. 2.5 software analysis (Applied Biosystems, Foster City, CA). The control cultivar "Harosoy" (Weiss and Stevenson 1955) used by Xu et al. (2002) was used for compatibility of results as a relative size reference (RSR) for the products in each gel. Haplotype assignment based on the combination of product sizes produced by the six cpSSRs followed the numbering system established by Xu et al. (2002). For the assay with the gmcp1 primer set alone, an RSR was used to categorize chloroplast haplotypes as either the same product size as Harosoy (0), or one base smaller than Harosoy (−1). All *G. max* chloroplast haplotypes other than #49 were reported to have the −1 result with the gmcp1 primer set (Xu et al. 2002).

Results

Soybean genotypes were analyzed to determine if they could be distinguished by distinct chloroplast haplotypes, as was done in a study of Asian soybean accessions (Xu et al. 2002). Six cpSSRs were amplified from DNA isolated from each soybean accession and compared to the cpSSR product sizes generated from the standard line Harosoy. The comparison to Harosoy was necessary because absolute product sizes were ambiguous due polymerase slippage causing the production of multiple bands from each primer set (Xu et al. 2002). Because of maternal inheritance of chloroplasts, we attempted, when possible, to use landraces and cultivars derived from selections to avoid loss of chloroplast information from hybridization events. Major North American soybean ancestors (Gizlice et al. 1994) were tested and assigned a haplotype group based on the numbering system of Xu et al. (2002). Similarly, representatives of the nine clusters and three outliers categorized based on nuclear polymorphisms (Brown-Guedira et al. 2000) were assayed for their chloroplast haplotype. All of these accessions (Table 1) contained chloroplast haplotype #49. Thus the ancestral North American soybean genotypes and representatives of the divergent clusters shared a common soybean chloroplast haplotype.

Although soybean lacks a standardized set of reference cultivars for molecular genetics experiments, certain genotypes are emerging as candidates. A subset of these cultivars used for molecular analyses (Shoemaker et al. 2002; Marek and Shoemaker 1997; Meksem et al. 2000; Zhu et al. 2003; Wu et al. 2004) were also assayed for chloroplast haplotype, and results are shown in Table 2. With the exception of Peking, all lines contained chloroplast haplotype #49. Peking, which had been previously assigned a type III chloroplast (Shoemaker et al. 1986; Xu et al. 2000) contained chloroplast haplotype #25, a haplotype group found in only 11% of Asian *G. max* cultivars examined earlier (Xu et al. 2002).

Because Peking is a source of resistance to SCN, other accessions with SCN resistance were assayed for chloroplast haplotype. After a set of plant introductions with resistance to multiple SCN HG types contributing to modern cultivars (plant introductions [PIs] 88788, 437654, 90763, and 209332) was analyzed, additional accessions with either extensive or limited resistance to SCN (Diers et al. 1997; Zhang et al. 1999) were characterized for their chloroplast haplotype. For these genotypes, the chloroplast haplotype was assayed at two levels: either an assay with all six cpssr primer sets to precisely define the chloroplast haplotype or with an RSR assay with the gmcp1 primer set to distinguish haplotypes similar to Harosoy (#49) from the other *G. max* chloroplast haplotypes (see *Materials and Methods*). Accessions classified as having multiple resistance (at least one resistance plus at least one moderate resistance) or limited resistance, generally contained chloroplast haplotype #25 or were excluded from the chloroplast #49 group by the gmcp1 assay (Table 3).

The PI 209332, which contained the chloroplast haplotype #49, and PIs 404166 and 548316 (Cloud), which contained gmcp1 products consistent with chloroplast haplotype #49, were the only exceptions in the set of multiple resistance lines. The SCN limited resistance lines contained either chloroplast haplotype #49 or a rare chloroplast haplotype. The results indicated that most soybean genotypes with resistance to multiple SCN HG types (32 of 35) were members of a divergent soybean chloroplast haplotype group. It was previously shown that the chloroplast haplotype groups #25 and #49 in cultivated soybeans could represent independent domestication events of soybeans from different *G. soja* gene pools or hybridization between cultivated and *G. soja* types (Xu et al. 2002). Because lines containing the type III chloroplast represented the group of genotypes with a haplotype other than the common #49 (Xu et al. 2002), we attempted to identify other type III lines present in the USDA GRIN to assay with the cpSSR primer sets. Besides Peking, one *G. max* accession (PI 224269 "Chasengoku 13," collected from Japan) that was known to be type III (Xu et al. 2000) was tested for chloroplast haplotype. This type III line contained chloroplast haplotype group #20. Haplotype #20 is the third most abundant haplotype group (5%), with a regional distribution concentrated in southern Japan (Xu et al. 2002). A small set of *G. soja* lines was also tested to determine their chloroplast haplotype. As expected, each of the *G. soja* lines tested contained different rare chloroplast haplotypes, with the exception of two lines with similar phenotypic attributes that were collected at the same location. In general, the chloroplast haplotype group corresponded to geographic locations as mapped by Xu et al. (2002).

Discussion

We set out to examine the genetic relationship at a broad level for various soybean lines using chloroplast haplotypes.

Table 1. North American soybean ancestors and plant introductions representative of divergent clusters that contain chloroplast haplotype #49

PI number	Cultivar	Origin	Maturity group[a]	Chloroplast type[b]	Cluster[c]
Ancestral					
153243	Dunfield	Jilin, China	III	I	C
157434	Illini	China	IV	I	I
189888	Mandarin (Ottawa)	Heilongjiang, China	I	I	B
548298	A.K. Harrow	China	III	I	I
548362	Lincoln	Unknown	III	nd	I
548391	Mukden	Liaoning, China	II	I	C
548406	Richland	Jilin, China	II	I	H
548445	CNS	Jiangsu, China	VII	II	H/F
548485	Roanoke	Jiangsu, China	VII	I	F
548488	S-100	Unknown	V	I	I
548493	Tokyo	Honshu, Japan	VII	I	ND
Clusters identified by Brown-Guedira et al. (2000)					
68508		China	II	ND	D
68600		China	II	ND	out
69507		China	I	ND	C
84657		South Korea	III	ND	B
87588		South Korea	IV	ND	J
91091		Jilin, China	II	ND	out
189930		Unknown	II	ND	A
291306 A		Heilongjiang, China	II	ND	F
361064		Unknown	II	ND	I
427088 B		Jilin, China	I	ND	K
437578		China	III	ND	out
467307		Jilin, China	I	ND	H
548360	Korean	North Korea	II	ND	A

Notes: Based on the numbering system of Xu et al. (2002). Maturity group designations were derived from USDA-ARS GRIN. Chloroplast haplotype was determined with six cpSSR primer sets.

[a] Day length requirements for soybean types grown in regions differing in latitude. Type I = most northern growing region and VII = most southern region.

[b] Chloroplast type from previous study (Shoemaker et al. 1986); ND = not determined.

[c] Cluster information from previous study (Brown-Guedira et al. 2000); ND = not determined; out = classified as outlier.

Most of the accessions tested were found to contain the common chloroplast haplotype (#49) and thus shared a common maternal ancestor. The broad classification of domesticated soybeans into relatively few chloroplast haplotype groups (Xu et al. 2002) has implications for incorporation of genetic diversity into elite soybean breeding programs. Genotypes containing chloroplast haplotype #25 or other rare *G. max* chloroplast haplotypes are easily identified with a single assay and potentially represent divergent soybean domestication events from genotypes with chloroplast haplotype #49. The results of this study revealed the presence of at least three chloroplast haplotypes

Table 2. Relationships among soybean germplasm resources for genomics, molecular genetics, and SNP discovery

PI no.	Cultivar	Origin	Maturity group[a]	Haplotype[b]	Chloroplast type[c]
209332		Hokkaido, Japan	IV	49	ND
290136	Noir 1		0	49	II
518671	Williams 82		III	49	I
548389	Minsoy		0	49	I
548402	Peking	Beijing, China	IV	25	III
548573	Harosoy		II	49	I
548655	Forrest[d]		V	49	ND

Notes: Maturity group designations were derived from USDA-ARS GRIN.

[a] Day length requirements for soybean types grown in regions differing in latitude. Type 0 = most northern growing region and V = most southern region.

[b] Based on the numbering system of Xu et al. (2002).

[c] Chloroplast type from previous studies (Shoemaker et al. 1986); ND = not determined.

[d] According to the pedigree of Forrest, Peking was used as a male parent (Hartwig and Epp 1973).

Table 3. Chloroplast haplotype for soybean PI lines with multiple (at least one resistance plus at least one moderate resistance) or limited resistance to SCN HG types

Multiple resistance to SCN			Limited resistance to SCN		
PI	RSR chloroplast haplotype	Chloroplast haplotype	PI	RSR chloroplast haplotype	Chloroplast haplotype
84751	−1		54591	0	
87631–1	−1		54620–2		49
88788	−1	25	79609	−1	
89772	−1		79693	−1	
90763	−1	25	89008	0	
200495	−1		89014	0	
209332	0	49	91138	0	
303652	−1		92720	0	
339868 B	−1		157430		49
399061	−1		398680	0	
404166	0		398682	0	
404198 A	−1	25	407944	−1	
404198 B	−1		408192–2	−1	
407729	−1		417094	−1	
416762	−1		423927		49
417091	−1		424595	−1	
424137 B	−1		437090	0	
437654	−1	25	437379	0	
437655	−1		437488	0	
437679	−1		437908	0	
437690	−1		438183	−1	
437725	−1		464888 A		49
437770	−1		548400 Patoka	0	
438342	−1		548415 Sooty	−1	
438489 B	−1		567285		25
438496 B Peking	−1				
438497 Peking	−1				
438498	−1				
438503 A	−1	25			
468915		25			
507471		25			
548316 Cloud	0				
548402 Peking (TN)	−1	25			
567491 A		25			
567516 C		25			

Notes: HG types from Diers et al. (1997); Zhang et al. (1999). An RSR was used to compare the cpSSR product sizes generated from Harosoy with products from the PIs; 0 = no difference, −1 = 1 bp difference. The chloroplast haplotype was determined with six cpSSR primer sets and assigned a haplotype number based on the work of Xu et al. (2002).

(#20, #25, and #49) in the USDA soybean germplasm collection.

We discovered an association between resistance to multiple HG types of SCN and an uncommon chloroplast haplotype. It was striking that 32 out of 35 accessions with resistance to multiple SCN HG types either were confirmed to contain chloroplast haplotype #25 or were shown to be excluded from the common haplotype #49 based on the size of a definitive primer set (gmcp1). The exceptions, PIs 209332, 404166, and 548316 (Cloud), may be sources of novel alleles for SCN resistance at previously mapped resistance loci (Cregan et al. 1999). Possession of the common chloroplast haplotype in these lines may also have been the result of undocumented hybridization events. Previous work examined the genetic relatedness of SCN-resistant PIs based on RFLPs in the nuclear genome. Consistent with our results, most genotypes with resistance to multiple SCN HG types broadly clustered together, independent from susceptible cultivars, although PI 209332 and 548316 clustered nearer to the susceptible group than the resistant group (Diers et al. 1997; Zhang et al. 1999). PI 404166 grouped loosely with the resistant lines in those experiments. Apparently, classification of germplasm based on chloroplast haplotype is also representative of nuclear diversity. Our broad classification of genetic relatedness based on chloroplast haplotypes adds an important component to classification of germplasm with similar phenotypic properties where an understanding of genetic diversity is important. Significantly, a single assay can be used to include or exclude a line from the common chloroplast haplotype group.

The underrepresentation of chloroplast haplotype #49 for lines containing multiple SCN resistances points to a

deficiency of genetic diversity within group #49 for extensive SCN resistance. The distinct *G. soja* ancestors which putatively contributed independently to domestication of soybeans representing group #49 and group #25 (Xu et al. 2002) may have differed in their resistance to SCN, or subsequent selection of other traits may have involved a loss of SCN resistance in group #49 or a gain of resistance in group #25. Thus we can speculate that the group of SCN-resistant accessions containing the uncommon chloroplast haplotype group descended from a common resistant ancestor and putatively share similar mechanisms of resistance. Our results do not suggest any genetic link between chloroplast haplotype and SCN resistance; rather, the data point to an ancestral divergence event that may have utility in identifying additional accessions with SCN resistance. It would be particularly appealing to characterize SCN resistance in *G. soja* genotypes containing chloroplast haplotype #25, as they potentially represent descendants of the undomesticated ancestors of the *G. max* group #25 (Xu et al. 2002).

We used six cpSSR primers sets to determine the chloroplast haplotype (Xu et al. 2002) for some soybean accessions and a high-throughput survey with a single primer set (gmcp1) to distinguish lines containing the common haplotype from other *G. max* haplotypes. The classification of chloroplast haplotypes can distinguish genotypes that are putatively derived from independent domestication events, and thus may have utility in incorporating broad genetic diversity into elite lines or determining the suitability of a genotype as a crossing partner for fine mapping of genes. The addition of a molecular component to the soybean GRIN system would add another dimension to the valuable phenotypic descriptions.

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